

**Dose-dependent pro- or anti-fibrotic responses of endometriotic stromal cells to interleukin-1 $\beta$  and tumor necrosis factor  $\alpha$**

**Running title:** Inflammation and endometriosis

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## Supplementary Note

### *Varying doses of IL-1 $\beta$ and/or TNF $\alpha$ in the present study*

Many previous in vitro experiments used very high levels of IL-1 $\beta$  and/or TNF $\alpha$  (ng- $\mu$ g/mL ranges) to elucidate the roles of these two proinflammatory cytokines in the pathophysiology of endometriosis.<sup>1-5</sup> However, it is not clear whether such high levels of IL-1 $\beta$  and/or TNF $\alpha$  could be involved in the pathophysiology of endometriosis. Previous studies reported much lower levels of IL-1 $\beta$  and/or TNF $\alpha$  in serum and peritoneal fluid of patients with endometriosis (pg/mL range).<sup>6-10</sup> Although proinflammatory cytokines are produced predominantly by activated macrophages, a previous in vitro study showed that peritoneal macrophages from patients with endometriosis produced TNF $\alpha$  in the pg/mL range following stimulation with LPS. High levels of IL-1 $\beta$  and/or TNF $\alpha$  (ng- $\mu$ g/mL ranges) in previous experiments<sup>1-5</sup> may not be recapitulated in the in vivo environment of patients with endometriosis.

Thus, first, we measured active IL-1 $\beta$  and TNF $\alpha$  secretion of endometriotic stromal cells, and menstrual endometrial stromal cells of patients with endometriosis and those of healthy control women, after stimulation with LPS or Poly I:C, to estimate potential pathophysiological ranges of IL-1 $\beta$  and TNF $\alpha$  in endometriotic tissues. In endometriosis, as in many other fibrotic disorders,<sup>11</sup> the inflammatory triggers remain unknown. Thus, in the present in vitro experiments, inflammation was non-specifically triggered by stimulation of innate immunity with either lipopolysaccharide (LPS) or polyinosinic:polycytidylic acid (Poly I:C).<sup>11</sup> Both toll-like receptor (TLR)-3 and -4 recognize dying/dead cells.<sup>12</sup> During menstruation, dying endometrial cells may activate TLRs, including TLR-3 and -4, in endometrial cells. Furthermore, studies have shown that sustained TLR signaling contributes to the development of many chronic inflammatory and autoimmune diseases.<sup>13, 14</sup> Thus, in the present study, inflammation was triggered by acute (4-h) and chronic (24-h) stimulation with either LPS or Poly I:C.

The present ELISA analyses suggested that TLR activation may induce low-grade local inflammation in endometriotic and menstrual endometrial stromal cells. According to the present ELISA analysis results as well as those of previous in vivo studies,<sup>6-10</sup> we further evaluated the effects of varying ranges of IL-1 $\beta$  and TNF $\alpha$  (pg/mL range) on fibrosis in endometriosis.

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## Supplementary Methods

### *Treatment of cells*

For enzyme-linked immunosorbent assay (ELISA) analyses, cells were incubated with either LPS (100 ng/mL), Poly I:C (10 µg/mL), or vehicle alone for 4 h or 24 h. Human recombinant IL-1β protein (R&D Systems, Lille, France) and TNFα protein (R&D Systems) were dissolved in phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA). LPS (Sigma-Aldrich), Poly I:C (Sigma-Aldrich), or adenosine triphosphate (ATP; Sigma-Aldrich) were dissolved in phenol red-free DMEM:Nutrient Mixture F-12 (DMEM/F-12) (Life Technologies).

### *Cytokine quantification*

Secreted IL-1β and TNFα levels in culture supernatants were assayed in duplicate and quantified by ELISA with DuoSet ELISA kits (R&D Systems) according to the manufacturer's instructions. The lower detection limits were 1.0 pg/mL (IL-1β) and 6.23 pg/mL (TNFα). Briefly, cells ( $3 \times 10^4$  cells per well) were plated in 24-well plates. After 48 hours, LPS, Poly I:C, or vehicle were added with 500 µL culture media (2% charcoal-stripped FBS) (Sigma-Aldrich) and incubated for 4 h or 24 h, and supernatants were collected. We utilized a well-established LPS or Poly I:C (1<sup>st</sup> signal; priming)/ATP (a damage-associated molecular pattern [DAMP]) (2<sup>nd</sup> signal; NLR activation) stimulation protocol to measure active IL-1β secretion after activation of the NLRP3 inflammasome.<sup>1</sup> Cells were incubated with 3 mM ATP for the last 15 min based on the method of Stoffels et al.<sup>1</sup> to measure active IL-1β secretion. Absorbance was read at 450/540 nm using a Multiskan microplate reader (Thermo Scientific). The concentration (pg/mL) was normalized to total protein content (pg/µg total protein) to control for unwanted sources of variation. Intra-assay precision was 4.3% (IL-1β) and 5.4% (TNFα), and inter-assay precision was 7.4% (IL-1β) and 8.9% (TNFα).

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## Supplementary Results

### *Effects of LPS or Poly I:C stimulation on IL-1 $\beta$ and TNF $\alpha$ secretion in endometrial and endometriotic stromal cells*

Neither LPS nor Poly I:C stimulation had a significant effect on IL-1 $\beta$  secretion at 4 h in M-ES-healthy (Supplementary Figure S1A). In contrast, after 24-h chronic stimulation with either LPS or Poly I:C, IL-1 $\beta$  secretion significantly increased in M-ES-healthy (Supplementary Figure S1B). Both LPS and Poly I:C stimulation significantly increased IL-1 $\beta$  secretion in M-ES-endo at 4 h (Supplementary Figure S1A) and 24 h (Supplementary Figure S1B). In both M-ES-healthy and M-ES-endo, both LPS and Poly I:C stimulation significantly increased TNF $\alpha$  secretion at 4 h (Supplementary Figure S1C) and 24 h (Supplementary Figure S1D). In ectopic endometriotic cells (Ectopic-ES), neither LPS nor Poly I:C stimulation had a significant effect on IL-1 $\beta$  (Supplementary Figure S1A) or TNF $\alpha$  (Supplementary Figure S1C) secretion at 4 h. In contrast, after 24-h chronic stimulation with either LPS or Poly I:C, IL-1 $\beta$  (Supplementary Figure S1B) and TNF $\alpha$  (Supplementary Figure S1D) secretion significantly increased.

**Supplementary Figure Legends**

**Supplementary Figure S1: Effects of LPS (100 ng/mL) or Poly I:C (10 µg/mL) stimulation for 4 h or 24 h on interleukin-1 $\beta$  (IL-1 $\beta$ ) (A, B) secretion or tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) (C, D) secretion in menstrual endometrial stromal cells of healthy fertile women (M-ES-healthy) and those of patients with endometriosis (M-ES-endo), and endometriotic stromal cells (Ectopic-ES)**

Numerical values are presented as the mean + SD.

\*:  $p < 0.05$  versus control (vehicle alone) within the same group.

M-ES-healthy: n=6

M-ES-endo: n=8

Ectopic-ES: n=10

**Supplementary Figure S2**

**Effects of IL-1 $\beta$  on cell proliferation of Ectopic-ES derived from deep infiltrating endometriosis versus ovarian endometriosis.**

Cells were incubated for 48 h at the indicated concentrations.

Percent cell proliferation was calculated as percent of vehicle control after 48-h treatment.

Numerical values are presented as the mean  $\pm$  SD.

C: control (vehicle alone)

Deep infiltrating endometriosis: n=8

Ovarian endometriosis: n=8

**Supplementary Figure S3: Effects of combinations of IL-1 $\beta$  and TNF $\alpha$  with or without TGF- $\beta$ 1 on mRNA expression of Col I (A, D, G), MMP-1 (B, E, H), and  $\alpha$ SMA (C, F, I) in M-ES-healthy (A-C), M-ES-endo (D-F), and Ectopic-ES (G-I).**

Cells were incubated at the indicated concentrations.

Expression levels of Col-I, MMP-1, and  $\alpha$ SMA mRNAs are given relative to the expression level of the reference gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Numerical values are presented as the mean + SD.

\*: p<0.05 versus control (vehicle alone).

#: p<0.05 versus TGF- $\beta$ 1 alone

Effects of the highest dose of IL-1 $\beta$  (100 pg/mL) on mRNA expression of Col I, MMP-1, and  $\alpha$ SMA in M-ES-healthy and M-ES-endo were excluded for further analyses, due to markedly reduced cell viability.

Because there were no significant differences in either Col I or  $\alpha$ SMA protein expression between vehicle-treated control after 48h and 96h, results of vehicle-treated control after 96h were not shown.

M-ES-healthy: n=8

M-ES-endo: n=16

Ectopic-ES: n=22

**Supplementary Figure S4: Effects of combinations of IL-1 $\beta$  and TNF $\alpha$  with or without TGF- $\beta$ 1 on Col I protein expression (A, C, E) and  $\alpha$ SMA<sup>+</sup> stress fibers (B, D, F) in M-ES-healthy (A, B), M-ES-endo (C, D), and Ectopic-ES (E, F).**

(A-F) The percentage of cells with Col I+ or  $\alpha$ SMA+ stress fibers after stimulation with IL-1 $\beta$  or TNF $\alpha$  alone or following TGF- $\beta$ 1 5 ng/mL stimulation in M-ES-healthy, M-ES-endo, and Ectopic-ES.

\*: p<0.05: versus control (vehicle alone)

#: p<0.05 versus TGF- $\beta$ 1 alone

Numerical values are presented as the mean  $\pm$  SD.

M-ES-healthy: n=8

M-ES-endo: n=16

Ectopic-ES: n=22

Effects of the highest dose of IL-1 $\beta$  (100 pg/mL) on protein expression of Col I and  $\alpha$ SMA in M-ES-healthy and M-ES-endo were excluded for further analyses, due to markedly reduced cell viability.

Because there were no significant differences in either Col I or  $\alpha$ SMA protein expression between vehicle-treated control after 48h and 96h, results of vehicle-treated control after 96h were not shown.

**Supplementary Figure S5:** Representative photomicrographs of double immunofluorescence staining for Col I and  $\alpha$ SMA in M-ES-healthy, M-ES-endo, and Ectopic-ES after stimulation with IL-1 $\beta$  and/or TNF $\alpha$  alone or following TGF- $\beta$ 1 5 ng/mL stimulation. Scale bar: 50  $\mu$ m.

M-ES-healthy: n=8

M-ES-endo: n=16

Ectopic-ES: n=22

Effects of the highest dose of IL-1 $\beta$  (100 pg/mL) on protein expression of Col I and  $\alpha$ SMA in M-ES-healthy and M-ES-endo were excluded for further analyses, due to markedly reduced cell viability.